

# Site-specific labeling of *Saccharomyces cerevisiae* ribosomes for single-molecule manipulations

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## ABSTRACT

**Site-specific labeling of *Escherichia coli* ribosomes has allowed application of single-molecule fluorescence spectroscopy and force methods to probe the mechanism of translation. To apply these approaches to eukaryotic translation, eukaryotic ribosomes must be specifically labeled with fluorescent labels and molecular handles. Here, we describe preparation and labeling of the small and large yeast ribosomal subunits. Phylogenetically variable hairpin loops in ribosomal RNA are mutated to allow hybridization of oligonucleotides to mutant ribosomes. We demonstrate specific labeling of the ribosomal subunits, and their use in single-molecule fluorescence and force experiments.**

## INTRODUCTION

Single-molecule methods have extensively investigated the mechanisms of translation in prokaryotes. By removing space and time averaging inherent to bulk systems, single-molecule approaches allow studies of heterogeneous and asynchronous systems. Protein synthesis is a multistep repetitive process that rapidly becomes asynchronous as ribosomes progress through multiple steps of elongation thus challenging application of conventional biochemical and biophysical techniques.

To overcome this obstacle, single-molecule fluorescence has been applied to probe interactions between ribosomes, tRNA and translation factors, and to monitor conformational dynamics of the ribosome. Introduction of the fluorescent dye pairs that can undergo Förster resonance energy transfer (FRET) into the ribosome and its ligands revealed conformational changes occurring in ribosome and tRNA as ribosome progresses through initiation, elongation and termination (1–4). FRET pairs incorporated into ribosomal subunits reported on the conformational and structural dynamics of the ribosomal particle and revealed new

steps and kinetic intermediates (5–8). Fluorescent labeling of the ribosomal subunits permitted continuous observation of ribosome dynamics through initiation followed by multiple rounds of elongation using single-molecule detection, avoiding the problem of rapid desynchronization of the ribosome population; these observations were only possible at a single-molecule level (7,9). Specific labeling of the ribosomal particles also allowed application of the force methods. Changes in mechanical stability of the mRNA:ribosome complexes in response to bound tRNA ligands revealed mechanism of Shine–Dalgarno clearance transition to elongation (10). Subsequent work by Bustamante group allowed observation of individual ribosome progression along mRNA (11). Thus, manipulation of bacterial ribosomes was central to allow detailed single-molecule investigation of mechanisms of prokaryotic translation.

Two general strategies for labeling ribosomal particles have been applied to prokaryotic ribosomes. The well-studied self-assembly of ribosomal particles from isolated protein and RNA allows introduction of the fluorescent labels into ribosomal proteins. Purified proteins are labeled using maleimide dye chemistry at genetically introduced single cysteine residues. Ribosomal particles are then assembled using the labeled proteins (12). Labeled versions of several ribosomal proteins, such as L1 or L11 can be incorporated to preassembled ribosomes that are missing the unlabeled versions of the proteins, created biochemically or genetically. Alternatively, metastable hairpins that readily anneal with labeled oligonucleotides can be genetically introduced into rRNA (13). Phylogenetic analysis guides the sites of mutation that do not disrupt ribosomal function. This allows placement of the fluorescent labels and molecular handles necessary for force measurements into ribosomal subunits. Genetic systems allow selection of pure populations of functionally mutant ribosomes that are subsequently labeled by hybridization with dye-linked oligonucleotides. Both labeling approaches have been used to explore dynamics of the bacterial ribosomal particle [Reviewed in (14)].

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Eukaryotic translational mechanism is highly complex and regulated. Translation initiation involves 5'-cap recognition, scanning and start codon selection. Alternative mechanisms allow distinct translational responses. Application of bulk methods has revealed the main steps of initiation, but precise molecular mechanisms remain obscure. The application of single-molecule methods will allow investigation of eukaryotic translation at single-molecule level at nanometer scale. Complex maturation and assembly process of eukaryotic ribosomes complicates labeling of the individual eukaryotic ribosomal proteins with subsequent incorporation into ribosomal particle. Here, we exploited an rRNA modification approach and describe the construction and characterization of yeast ribosomes bearing labeling hairpins in surface exposed regions of rRNA. We demonstrate specific labeling of yeast ribosomes with fluorescently labeled oligonucleotides, and application of these ribosomes to single-molecule fluorescence and force experiments.

## MATERIALS AND METHODS

### Strains, media, reagents and molecular methods

*Escherichia coli* strain DH5 $\alpha$  was used for cloning and to amplify plasmids. Yeast media contained 2% galactose instead of glucose; drug concentrations were as follows: doxycycline, 10  $\mu$ g/ml; and hygromycin B, 300  $\mu$ g/ml. Transformations of yeast strains were performed according to an alkaline cation protocol and yeast cells were grown at 30°C. Yeast strain pJD1314 lacking RDN operons (*MATa ade2-1 ura3-1 trp1-1 his3-11 leu2-3, can1-100  $\Delta$  rDNA::his3::hisG + [pNOY353 (GAL7-RDN37 RDN5 TRP1 2 $\mu$  AMP)] [L-A HN MI]*) and pJD694 plasmid containing RDN35 under tetracycline repressible promoter were kindly provided by J. D. Dinman (15). Total yeast RNA was isolated by acid phenol extraction and treated with RNase-free DNase (QIAGEN, Valencia, CA, USA). The Titan One-Tube RT-PCR system (Roche) was used for reverse transcription-PCR (RT-PCR).

### Ribosome mutagenesis

Mutations were introduced into 18S and 25S rRNA of *Saccharomyces cerevisiae*. Two-step megaprimer PCR was used to incorporate labeling hairpins into pJD694 (*URA3*) plasmid carrying 35S rRNA under a tetracycline repressible promoter. Corresponding primers are listed in Supplementary Table S1. The resulting plasmids were transformed into pJD1314 *S. cerevisiae* strain, where RDN operons were deleted and supplied from 2  $\mu$ m *TRP1*-carrying plasmid under galactose inducible promoter (15). Transformants were grown on -Ura, -Trp, Gal, Dox media for 10 days and then streaked twice on the -Ura, -Trp, Gal media for 3 days to establish stable expression of the mutant rRNA. Subsequently, strains were replica plated on -Ura media, to turn off transcription of wild-type rRNA. Strains were replica-plated four more times on the -Ura media to induce spontaneous loss of the *TRP1*-containing plasmid. The resulting strains were streaked for single

colonies and simultaneously replica-plated on -Ura, Dox and -Ura, Hyg media. To select against recombinant plasmids and strains expressing both wild-type and mutant rRNA, the recessive hygromycin B resistance mutation has been introduced into 18S rRNA gene carrying hairpin insertion. Thus strains expressing wild-type or mixture of the wild-type and mutant rRNA were unviable in the presence of the drug. After 2 days, colonies were replica-plated again on the same media and incubated for 4 more days. Yeast growing on -Ura, Hyg but not growing on -Ura, Dox media were re-streaked for single colonies on -Ura, Dox media and selection on -Ura, Dox and -Ura, Hyg was repeated. The resulting strains were grown in -Ura media for 3 days, and 3  $\mu$ l of yeast suspension was spotted onto either -Ura, Gal, Dox; -Ura; -Ura, Dox; or -Ura, Hyg media and grown for 2 days to select against recombinant strains and to assess the efficiency of pGAL plasmid elimination. The phenotype was re-confirmed by subsequent replica plating on the same medias. Pure cell cultures that were viable in -Ura and -Ura, Hyg but not in both -Ura, Dox and -Ura, Gal, Dox were used to obtain total nucleic acids by acid phenol extraction. Segments of interest were amplified by RT-PCR and sequenced to confirm presence of the mutant rRNA (Supplementary Figure S1). The 80S ribosomes were purified from positive strains, rRNA was isolated by phenol extraction and presence of the insertion was confirmed again by the sequencing of RT-PCR product.

### Preparation of ribosomal subunits

Yeast ribosomes were prepared as described before with the following modifications (16). Fresh stock plate was used to inoculate 6ml liquid YPAD culture. Cells were grown with shaking at 250 rpm till the culture reached an OD<sub>600</sub> = 1.0. The resulting culture was used to inoculate 12 l of YPAD in six 6-l flasks. Cells were grown to OD<sub>600</sub> = 0.75 and were collected by centrifugation. Cell mass was washed twice with 500 ml of 20 mM HEPES-KOH pH 7.4, 100 mM KOAc, 10 mM Mg(OAc)<sub>2</sub>, 1 mg/ml heparin and 2 mM dithiothreitol (DTT). Finally, ~20 g of cells were mixed with 40 ml of 20 mM Hepes-KOH pH 7.4, 100 mM KOAc, 10 mM Mg(OAc)<sub>2</sub>, 1 mg/ml heparin, 2 mM DTT and Roche complete protease inhibitor tabs, and frozen in liquid nitrogen. Cells were lysed by Waring blender method. The lysate was clarified by centrifugation in SW28 rotor for 30 min at 15 000 rpm. The supernatant was collected and centrifuged again at the same conditions. Lysate was overlaid on top of 2.5 ml of sucrose cushion [20mM HepesKOH pH 7.4, 500mM KOAc, 10mM Mg(OAc)<sub>2</sub>, 1 mg/ml heparin, 2mM DTT, Roche complete protease inhibitor, 1M sucrose] in polycarbonate tube for 70Ti rotor. Ribosomes were precipitated by centrifugation in 70Ti rotor for 140 min at 55 000 rpm. Pellets were re-suspended in ~20 ml of 20 mM Hepes-KOH pH 7.4, 500mM KOAc, 10mM Mg(OAc)<sub>2</sub>, 1 mg/ml heparin and 2mM DTT, and spun through the sucrose cushion again. Ribosomes were re-suspended in 20 mM Hepes-KOH pH 7.4, 500mM KOAc, 10mM Mg(OAc)<sub>2</sub>, 2mM DTT, and loaded on

top of 5–20% sucrose gradients (50 mM Hepes–KOH pH 7.4, 500 mM KCl, 10 mM MgCl<sub>2</sub>, 0.1 mM EDTA and 2 mM DTT) prepared in SW28 tubes. Gradients were spun at 28 000 rpm for 7.5 h and fractionated with ISCO gradient fractionators. Peaks corresponding to 40S and 60S ribosomal subunits were collected and concentrated using Amicon 20, 100 000 molecular weight cutoff (MWCO) spin concentrators to the volume of <1 ml. Then subunits were diluted to 12.5 ml with ribosome storage buffer and concentrated again to the volume of 200–500 µl, aliquoted and frozen in liquid nitrogen. To confirm homogeneity of the ribosomal population, rRNA was extracted and segments containing labeling hairpins were amplified by RT–PCR and sequenced.

### Ribosome labeling and gel shift assays

Binding assays were carried out with 2 nM Cy3-labeled oligonucleotides and 2 nM purified ribosomes preheated at 37°C for 5 min and incubated for 5 min at 30°C (unless indicated otherwise) in Tris–polymix buffer supplemented with 10 mM MgCl<sub>2</sub> (2). After incubation, one-tenth the volume of 1.75 M sucrose was added. Reactions were resolved on native composite gels containing 0.5% agarose, 2.75% acrylamide, 25 mM Tris–acetic acid pH 7.5, 6 mM KCl, 12 mM MgCl<sub>2</sub>, 1 mM DTT and 1% sucrose (17), scanned with Typhoon scanner and quantified with ImageJ. Dissociation equilibrium ( $K_D$ ) and kinetic rate ( $k_{off}$ ) constants were estimated using single binding site equilibrium binding model and exponential decay model, respectively. Data were fitted using GraphPad Prism.

### Single-molecule measurements

H39 or H44 40S subunits were labeled with Cy3-containing and H63 60S mutant was labeled with Cy5-containing oligonucleotides, respectively. The 80S ribosomal complexes were assembled on the GGUAAC ACGCAUAUGUAAUACGACUCACUAUAGGA AUGCUCUCUCUCUCUAUGCUCUCUCUCUCUCUCUCUCUC mRNA containing covalently linked biotin on the 5'-end in polymix buffer supplemented with 12 mM MgCl<sub>2</sub>. The microscopic chambers derivatized with PEG 5000 and biotin–PEG 5000 were prepared as described before (2). Twenty microliter of 1 nM neutravidin was flown into the ~7-µl microscopic chamber and incubated for 5 min. The chamber was washed with 200 µl of 50 mM Tris–acetic acid pH 7.5, 100 mM KCl and with 200 µl of polymix buffer with 12 mM MgCl<sub>2</sub>. Labeled ribosomal complexes were delivered to the surfaces and incubated for 5 min. The unbound complexes were washed out with 100 µl of polymix buffer supplemented with 12 mM MgCl<sub>2</sub>, 1 mM Trolox and oxygen scavenging system consisting of 2.5 mM protocatechuic acid and 10 nM protocatechuate-3,4-dioxygenase (18). Fluorescence was monitored by using homebuilt prism-based TIRF microscope.

For single-molecule nano-manipulations anti-digoxigenin antibodies were covalently linked to the

surface of the 1 µm polystyrene carboxylate-modified beads via 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride cross-link (19). The 40S subunits were labeled with Cy3 oligonucleotide-containing digoxigenin on the 3'-end. The 80S:mRNA ribosomal complexes were assembled on the surface of the microscopic slides as in single-molecule fluorescence experiment. The 1-µm polystyrene beads in polymix buffer supplemented with 1 mM Trolox and oxygen scavenging system consisting of 2.5 mM protocatechuic acid and 10 nM protocatechuate-3,4-dioxygenase were delivered to the microscopic chamber and incubated for 5 min. The mechanical stability of the mRNA:ribosome complexes was measured on homebuilt optical-trap/objective-based TIRF instrument at 13 pN × sec<sup>-1</sup> loading rate.

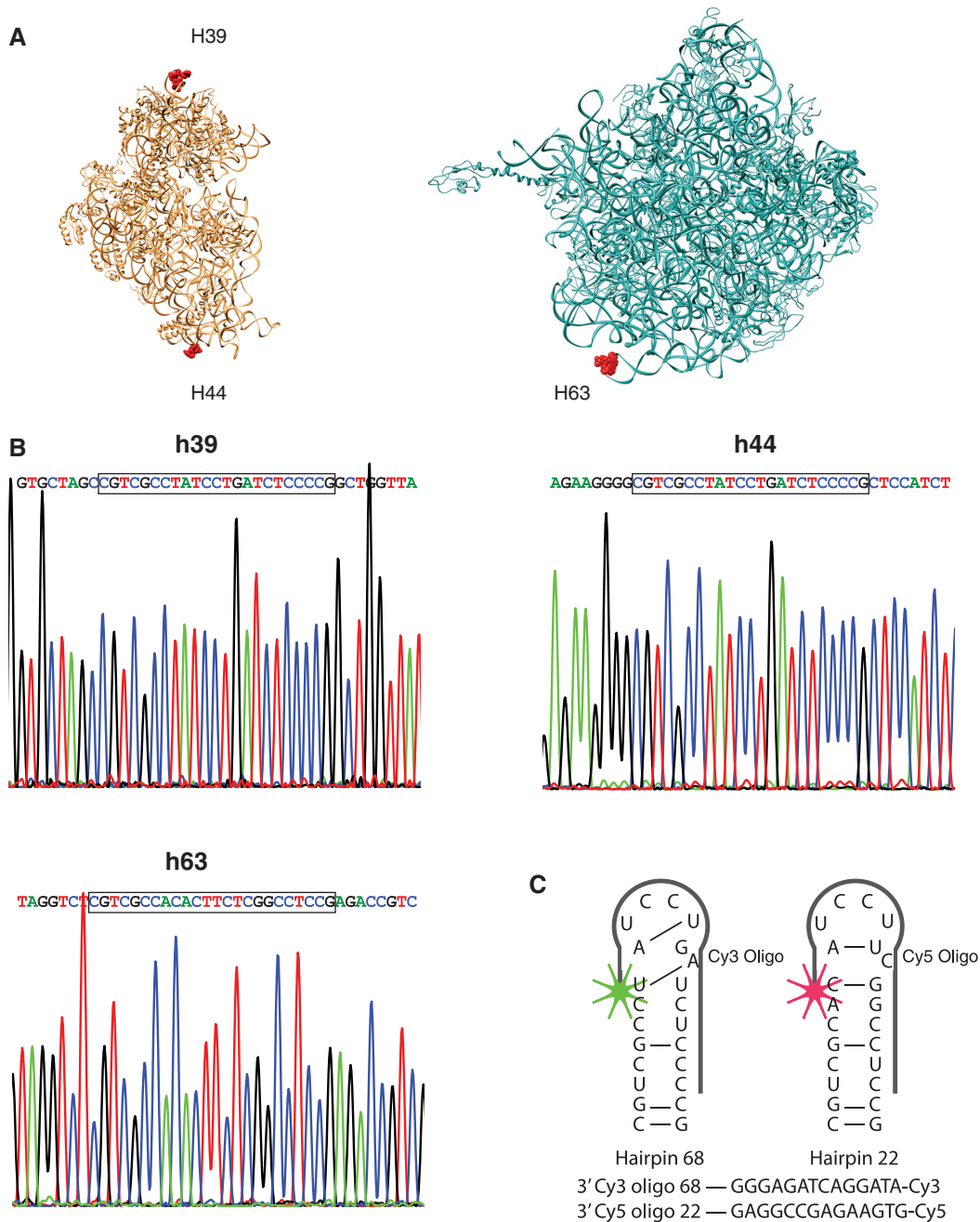
## RESULTS

### Labeling site design and strain construction

Labeling hairpins were inserted into phylogenetically variable regions of *S. cerevisiae* 18S, 5.8S and 25S rRNA (20) by cloning 23 nucleotide extensions that replaced surface-exposed rRNA loops. Ten sites were selected for mutagenesis in the initial approach: helices 39, 41 and 44 of 18S rRNA, helix 3 of 5.8S rRNA and helices 33, 38, 47, 63, 78 and 101 of 25S rRNA (Supplementary Figures S2 and S3). The sequence of the rRNA insertion forms a stable, partially mismatched helical extension that would readily unfold and hybridize with an oligonucleotide to achieve efficient rRNA labeling (13). Hairpin type 68 was inserted in to 18S rRNA and hairpin type 22 was inserted into 5.8S and 25 S rRNA.

Yeast rRNA mutagenesis was based on the system developed by Dinman and coworkers (15,21). The insertions were cloned into 35S rRNA-carrying plasmid and transformed into JD1394 strain. In this strain, RDN operons were deleted and 35S rRNA was expressed from pNOY353 plasmid under control of galactose inducible promoter (22). Expression of the mutant rRNA and loss of the wild-type plasmid was established as described in 'Materials and Methods' section. Strains bearing mutations at positions H39 and H44 of 18S rRNA and H63 of 25S rRNA were isolated (Figure 1). Strains bearing mutations at other positions could not be isolated due to high recombination rates. It is possible that these mutants could be obtained in the future by using different genetic approach. Presence of mutant ribosomes was confirmed by sequencing rRNA from purified ribosomes and only mutant rRNA was detected (Figure 1B). Labeling hairpins did not affect viability of *S. cerevisiae* as doubling times and viability of the mutants was comparable to wild-type strain as was demonstrated by the growth curves and dilution spot assays (Supplementary Figure S4).

The currently employed genetic system involves long and complicated selection scheme, which complicates high-throughput rRNA mutagenesis. Possibly future modifications such as use of the tighter promoter regulation and yeast strains with suppressed recombination machinery may simplify selection protocol. Also, introduction of the recessive hydromycin B resistance

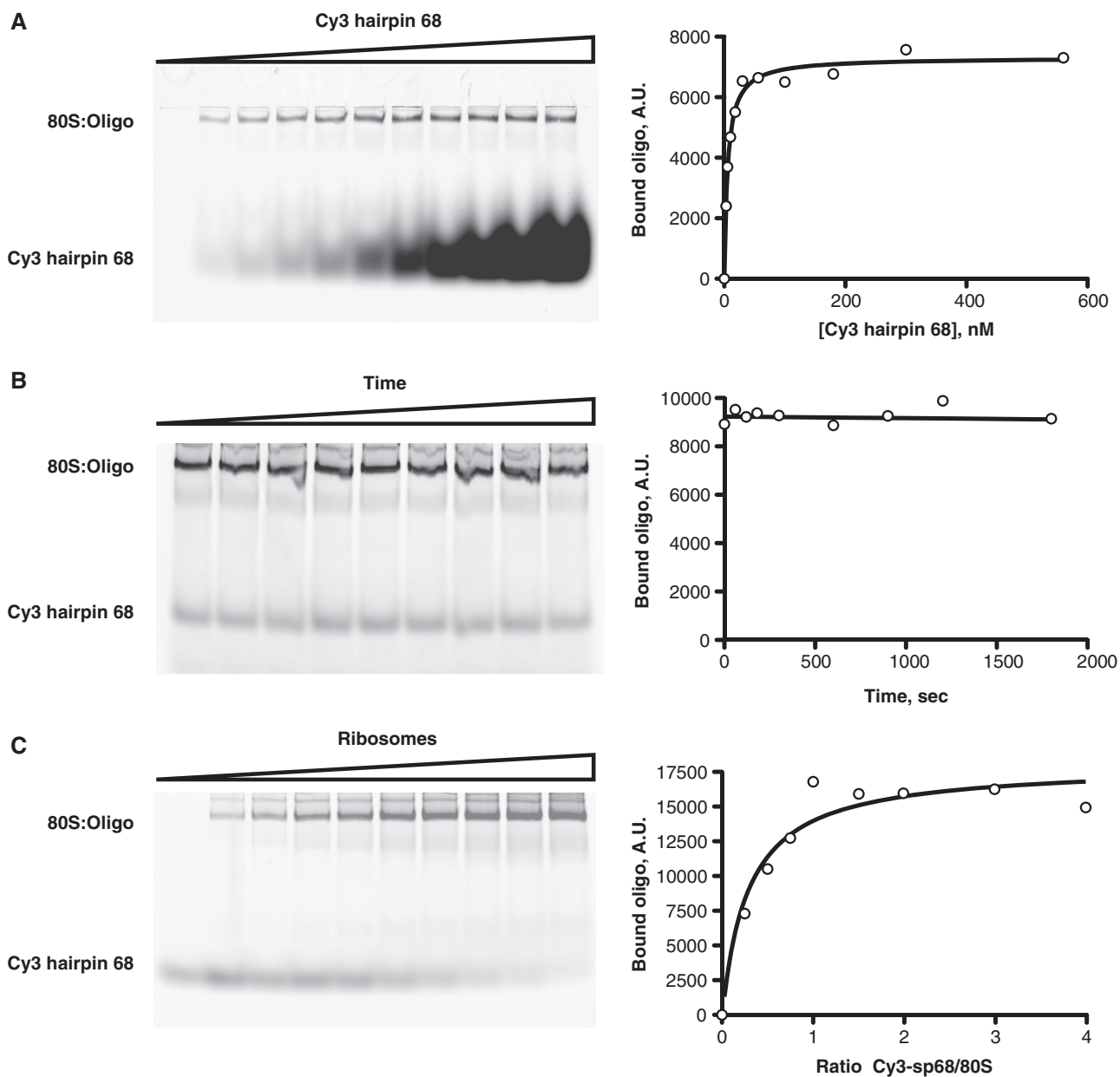


**Figure 1.** (A) Position of the labeling sites in yeast modeled on the 3D structure of *E. coli* ribosome as viewed from inter-subunit interface. Small subunit is shown in wheat and large subunit is in teal. Surface-exposed loops replaced by hairpins are shown in red. H39 is located on the top of the head and H44 is proximal to the spur of the small subunit. H63 is at the bottom of the as viewed from inter-subunit interface. (B) rRNA from ribosomes bearing hairpin insertions was amplified by RT-PCR and sequenced. (C) Labeling hairpins and labeling oligonucleotides.

markers close to the proposed mutation site (i.e into 25S rRNA) may facilitate selection of the mutant strains by reducing number of recombinant strains lacking hairpin insertion but carrying resistance marker. Alternatively, mutant rRNA could be expressed in the presence of the wild-type molecule. The inserted hairpin could be used to selectively place a purification tag on the mutant ribosomal particles, which could be used for separation of the mutant and wild-type ribosomes, and thus eliminating a need for selection procedure.

### Ribosome labeling

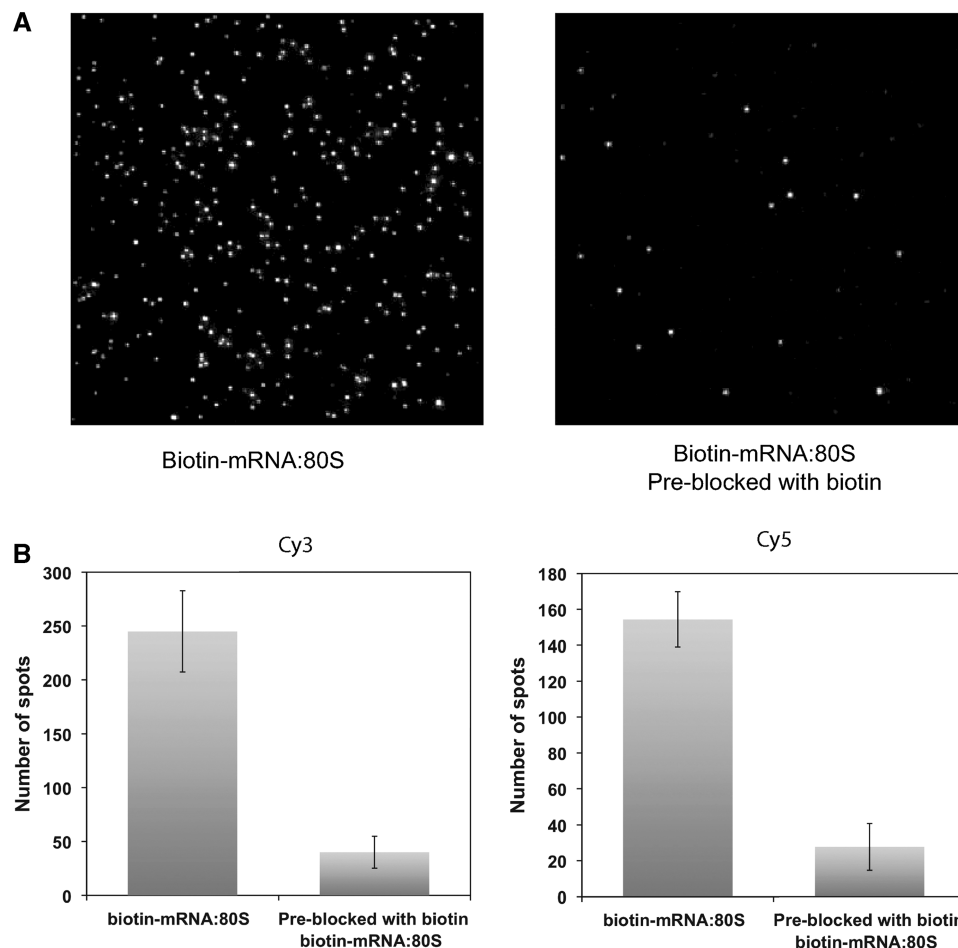
The ability of mutant yeast ribosomes to be labeled using oligonucleotides was confirmed biochemically. Mutant ribosomes containing rRNA expressed from pJD694 plasmid containing labeling hairpins in helices 39 and 44 of 18S rRNA and helix 63 of 25S rRNA were isolated as pure populations. Wild-type ribosomes were also expressed and isolated using this system. To test for binding of complementary oligonucleotide to the designed ribosomal extension and to optimize annealing



**Figure 2.** (A) Four nanomolar of H44 80S ribosomes were incubated with increasing concentrations of 5'-Cy3-sp68 oligonucleotide. Reactions were resolved on composite gel and apparent  $K_D$  was found to be 5 nM. (B) Labeled ribosomes were chased with 10-fold excess of dark sp68 oligonucleotide and incubated for up to 30 min at 30°C. No dissociation of the labeling nucleotide was detected. (C) The 1:1 labeling of the ribosomes with 5'-Cy3-sp68 oligonucleotide could be achieved by increasing incubation time to 15 min. Ten nanomolar ribosomes were incubated with increasing concentrations of 5'-Cy3-sp68 oligonucleotide. A 90–100% labeling efficiency could be reached at 1:1 ratio of the fluorescently labeled nucleotide to the ribosomes.

conditions, we performed hybridization with Cy3- and Cy5-labeled DNA oligonucleotides targeting hairpins in 18S and 25S rRNA, respectively; binding of oligonucleotides to ribosomal subunits was monitored by native composite gel electrophoresis (Figure 2A). The apparent  $K_D$  derived from these gel experiments for oligonucleotide hybridization to mutant ribosomes (5 nM) was similar to values observed for prokaryotic ribosomes (13). Binding of the oligonucleotides was specific for the appropriate complementary hairpin in the mutant ribosomes, as no complex formation was observed with subunits missing

the target hairpin (data not shown). The oligonucleotide–ribosome complexes were kinetically stable; dissociation rates were probed using chase experiments with excess unlabeled oligonucleotide. No dissociation of the Cy3 oligonucleotide was observed in 30 min (Figure 2B), with  $k_{off}$  estimated to be below  $7 \times 10^{-5} \text{ s}^{-1}$ . Thus the relatively high apparent  $K_D$  is defined by slow rate of the oligonucleotide association reaction. As with prokaryotic ribosome–oligonucleotide complexes, this indicates that oligonucleotide binding is sufficiently tight for single-molecule manipulations that are conducted in



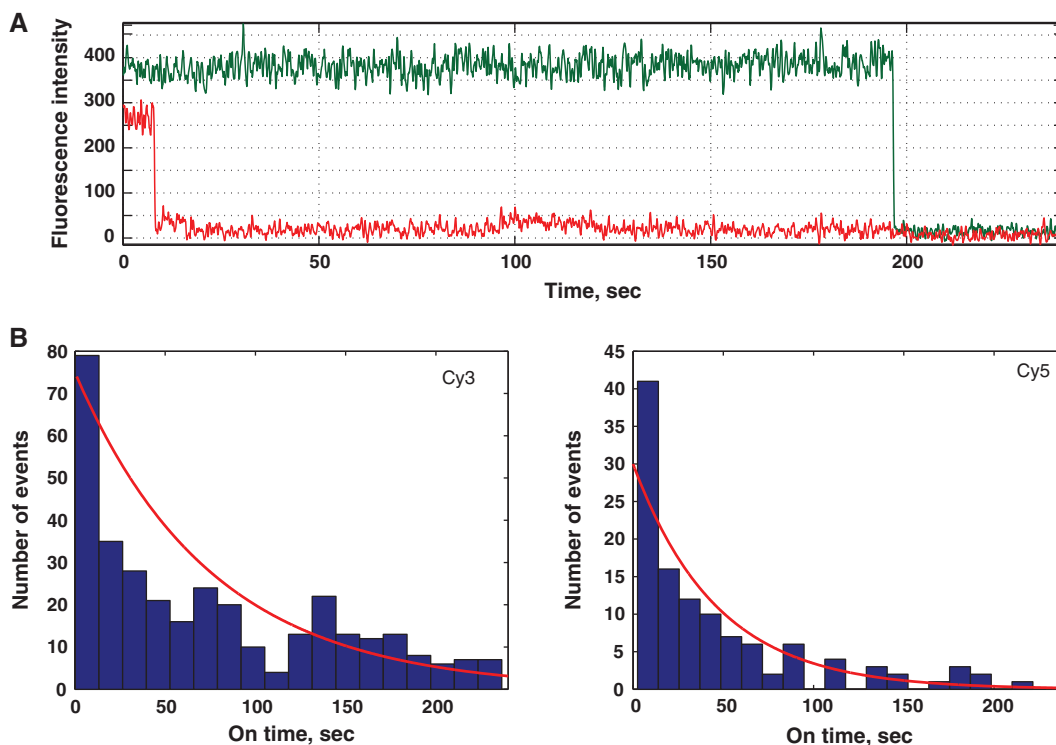
**Figure 3.** (A) Single-molecule fluorescence of biotin-mRNA:ribosome complexes immobilized on the surface of the quartz slide via biotin:neutravidin interactions. Ribosomes were labeled on the 40S subunit at H44 using Cy3-labeled nucleotide as described in the text. Fluorescence was excited by total internal reflectance illumination at 532 nm and detected using an EM-CCD camera. Preincubation of the slide with biotin abrogates complex binding. (B) Mean spot number in six fields of view.

picomolar to nanomolar concentration range. Increasing the hybridization temperature to 42°C, cycling labeling temperatures and higher salt concentrations improved apparent binding affinity but adversely affected ribosome stability (data not shown). Using prolonged incubation times up to 15 min at 37°C and 15 min at 30°C, we were able to label the small ribosomal subunit efficiently at 1:1 molar ratio (Figure 2C).

#### Selective immobilization and single-molecule observation

We next explored whether oligonucleotide-labeled yeast ribosomes can be detected using single-molecule fluorescence or force methods. H39 or H44 40S subunits were labeled with Cy3 and H63 60S mutant was labeled with Cy5 oligonucleotides. The 80S ribosomal complexes were assembled on biotinylated mRNA as described in the 'Materials and Methods' section. These assembled 80S:mRNA complexes were immobilized onto quartz slides derivatized with PEG 5000 and biotin-PEG 5000 via biotin–neutravidin interactions. Fluorescently labeled ribosomes were then detected as fluorescent spots within a field of view. On average, 240 Cy3 and 150 Cy5 spots were detected in  $\sim 100 \mu\text{m}^2$  field of view when 20  $\mu\text{l}$  of 1 nM

ribosomes were delivered to the chamber. Immobilization was selective and specific and was abrogated either by pre-incubation of the surfaces with 0.1  $\mu\text{M}$  of free biotin or by omitting biotinylated mRNA (Figure 3). Pre-incubation with biotin resulted in more than 5-fold reduction in number of immobilized molecules. The dye intensity histograms have a single peak at  $\sim 500$  and 300 AU for Cy3 and Cy5, respectively (Supplementary Figure S5). The single peaks in dye intensity histograms together with prevalence of the single photobleaching events ( $\sim 90\%$  of all events) in fluorescence time traces indicate the 1:1 stoichiometry of dye-labeled oligonucleotide to immobilized ribosome (Figure 4A). Fluorescence traces that exhibited a single photobleaching event and were not limited by the end of the movie were used to calculate dye lifetimes for continuous single-molecule emission. Lifetimes of individual dye molecules were quantified and were found to be 75.5 and 42.5 s for Cy3 and Cy5, accordingly. Translation *in vitro* is approximately an order of magnitude slower than *in vivo* and occurs at the rate of  $\sim 0.1$ –1 amino acid per second. Observed half-lives of the fluorescent dyes will permit continuous fluorescence observation on the time-scales up to



**Figure 4.** (A) Single-molecule fluorescence trace from biotin-mRNA: Cy3-40S: Cy5-60S ribosomal complexes. (B) Lifetimes of the Cy3 and Cy5 dyes on the 40S and 60S ribosomal subunits.

several minutes, and should be sufficient to continuously monitor initiation and elongation processes.

Ribosome-bound fluorophores were not quenched when compared to the dye-labeled oligonucleotides. Signal stability of ribosome-bound fluorophores (defined as a ratio of the background-corrected fluorescence intensities to the SD of the signal intensity) was found to be 7.3 and 5.2 for Cy3 and Cy5, respectively. This fluorescence intensity and signal stability is sufficient for unambiguous identification of the ribosomal particles and change in relative fluorescence intensity in single-molecule experiments. The fluorescently labeled yeast ribosomal subunits could be colocalized to demonstrate 80S complex formation (an example is shown in Figure 4). These results further confirm that fluorescent labeling does not affect ribosome activity.

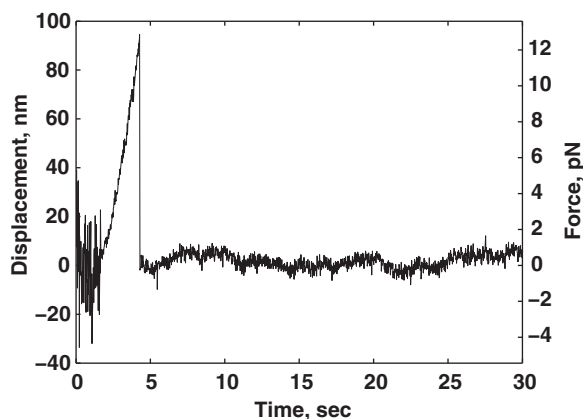
To test suitability of the labeled ribosomes for single-molecule nano-manipulations, H39 ribosomal subunits were labeled with 5'-Cy3-sp68-3'digoxigenin oligonucleotide and 80S:mRNA complexes were immobilized on the surface via biotin:neutravidin interactions. The 1- $\mu\text{m}$  polystyrene beads covered with covalently linked anti-digoxigenin antibodies were tethered to the ribosomal complexes via digoxigenin:antibody interactions. Thus, ribosomal complexes were stretched between the slide surface and the bead. To favor formation of the single tether between surface of the chamber and the bead, the concentration of ribosomal complexes delivered to the microscopic chamber was decreased by 10-fold to 100 pM. This resulted in corresponding 10-fold reduction in the number of the fluorescent spots.

At these conditions, formation of the ribosome:mRNA:bead complexes was limited by the rate of bead attachment. Between 10 and 30 attached beads were observed in the 1000- $\mu\text{m}^2$  view field. Dye fluorescence was used to validate complex composition. Upon observing single photobleaching indicating a single link between the slide surface and the bead, the bead was captured by an optical trap. The optical trap was used to apply constantly increasing force until the ribosome:mRNA complex ruptures (Figure 5).

## CONCLUSIONS

We successfully introduced hairpins into rRNA of small and large ribosomal subunits of *S. cerevisiae*. Strains bearing rRNA insertions are viable with mutant rRNA as the sole source for ribosomes. Mutated ribosomes were purified as pure populations and presence of the inserted hairpin was confirmed by rRNA sequencing. Ribosomal particles were tagged by annealing labeled oligonucleotides at physiological conditions to the inserted rRNA hairpins. The utility of these labeled yeast ribosomal subunits for single-molecule fluorescence and force measurements was shown.

Our labeling approach has several advantages compared to other methods. The use of labeled proteins and *in vitro* assembly for site-specific labeling of yeast ribosomes is complicated by the complex maturation and assembly process of eukaryotic ribosomes. Incorporation of the labeling sites into ribosomal proteins followed by reassembly of the ribosomal



**Figure 5.** Rupture trace. mRNA:80S ribosomal complexes are stretched between surface of the microscopic slide and polystyrene bead. Prior to measurement, the bead fluctuates around the attachment point. Bead capturing by an optical trap suppresses fluctuations. The piezoelectric stage is used to move surface of the slide, thus pulling the bead out of the focus of the trap. Bead displacement generates force linearly proportional to the displacement directed to the center of the optical trap. The force is increased until the complex ruptures.

particle may result in heterogeneous population of the ribosomal particles due to missing or misfolded/misassembled components. Hybridization of the labeled nucleotide to rRNA hairpins is free of such potential complication. Inserted hairpins form stable heteroduplex with labeled DNA oligonucleotides. Ribosomes could be labeled at 1:1 ratio with the corresponding oligonucleotide. The kinetic stability of the ribosome oligonucleotide interactions ( $k_{\text{off}} < 7 \times 10^{-5} \text{ s}^{-1}$ ) allows ribosome handling and manipulation at nanomolar and picomolar concentration range. The low dissociation rate constant permits prolonged fluorescence and force experiments where observations are not limited by stability of the oligonucleotide ribosome interactions. The labeled ribosomal complexes could be selectively immobilized on the surfaces via biotinylated mRNA and fluorescence from single fluorescent dyes could be observed.

These labeled yeast ribosomes will permit future application of single-molecule fluorescence methods to the study of eukaryotic translational mechanism. Labeled ribosomal subunits will allow precise tracking of arrival and departure of the subunits during initiation and termination processes. FRET between ribosome and ribosomal ligands will report on arrival and departure of those ligands, as well on conformational changes associated with reactions facilitated by those ligands. Intraribosome FRET will report on dynamics of the ribosomal particle.

Genetically introduced hairpins can also be used for simultaneous labeling of eukaryotic ribosome with fluorescent dyes and molecular handles for force experiments. Molecular handles such as digoxigenin and biotin provide ability to manipulate ribosomal complexes at nanoscale, while fluorescent dyes report on the complex composition and conformation. Incorporation of the labeling sites into ribosomal rRNA rather than into ribosomal proteins is potentially beneficial for force experiments. Testing of

mechanical stability of the ribosomal complexes requires application of relatively high forces (up to tens of piconewtons) that may result in dislocation of the tagged protein, rather than disruption of the rupture of the tested interactions. rRNA forms backbone of the ribosomal particle and cannot be dislodged. Notably, rRNA labeling approaches used here have been successfully used for force measurements in prokaryotes. Force experiments may reveal molecular mechanism of mRNA: ribosome interactions and can probe the scanning and codon recognition processes that imply movement of the ribosome along mRNA.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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